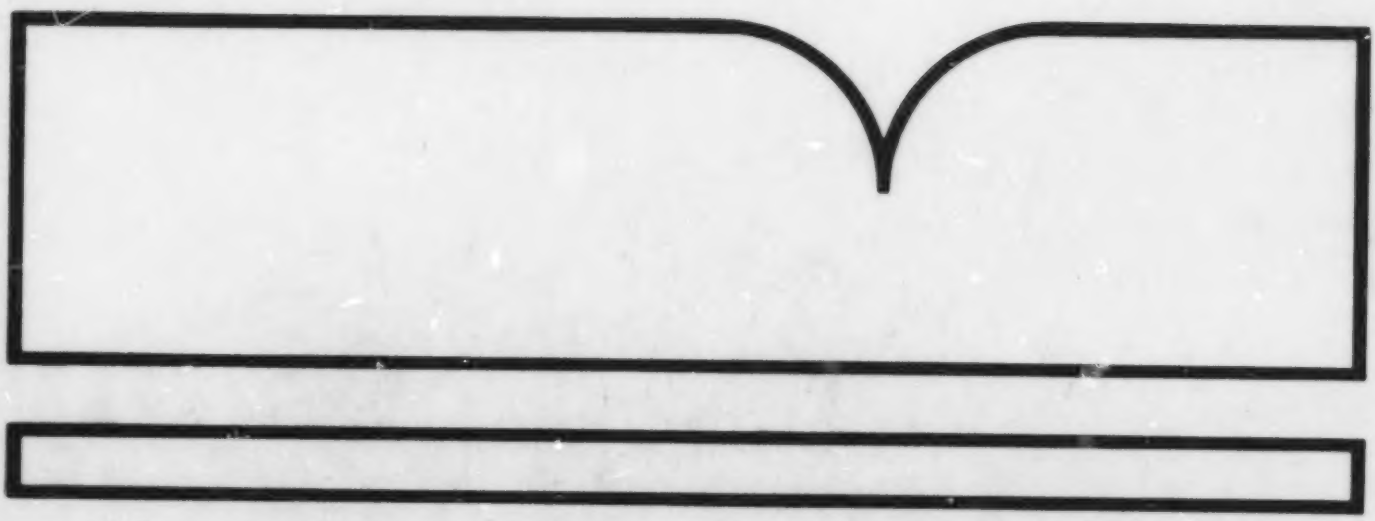
PB81-230880

Detection of Giardia Cysts in Drinking Water State-of-the-Art

(U.S.) Health Effects Research Lab.
Cincinnati, OH

Jul 81



U.S. Department of Commerce
National Technical Information Service

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/D-81-258	2. Book Chapter	3. REC PB81-230880
4. TITLE AND SUBTITLE Detection of Giardia Cysts in Drinking Water: State-of-the-Art		5. REPORT DATE July 1981 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Walter Jakubowski		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. EPA Health Effects Research Laboratory 26 W. St. Clair St. Cincinnati, OH 45268		10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Same		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE

15. SUPPLEMENTARY NOTES
To be published as a book chapter in: Giardia and Giardiasis, Plenum Press, June 1982

16. ABSTRACT
Methodology for detecting Giardia cysts in water samples is described. Problems in the collection and processing of samples and in the identification of cysts are discussed. The results of a methodology workshop are presented and a reference method is proposed.

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS *(This Report)* Unclassified	21. NO. OF PAGES
	20. SECURITY CLASS *(This page)* Unclassified	22. PRICE

DRAFT

EPA-600/D-81-258
Book Chapter

DETECTION OF *GIARDIA* CYSTS IN DRINKING WATER: STATE-OF-THE-ART

by

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1. INTRODUCTION

The detection, identification, and enumeration of microbiological contaminants, and in the present discussion specifically Giardia cysts in water supplies, is of concern to workers in a number of different disciplines. The microbiologist and the epidemiologist need to know the source of contamination, the level of contamination, the relationship to indicator organisms, and the reservoirs of infection. The engineer and the utility operator need to know how to remove Giardia or how to inactivate it by water treatment. Those responsible for administering drinking water programs want to know where and when the organism occurs in water, the suitability and availability of monitoring methods, and whether or not maximum contaminant levels, treatment requirements or guidance should be developed.

This information is needed on this pathogen because waterborne outbreaks continue to occur (Craun, 1979) and those in Camas, Washington (Kirner et al., 1978) and Berlin, New Hampshire (Lippy, 1978) in particular have demonstrated that the coliform standard cannot be relied upon to indicate the safety of a water supply with respect to Giardia. The possibility that other organisms or compounds could act as alternate indicators of Giardia contamination remains to be investigated. The application of cultural techniques to provide answers to the questions being posed is not now possible for Giardia in drinking water. In addition, certain basic information (e.g., how to determine species of origin, viability, or infectivity) necessary for the interpretation of results obtained through microscopic examination is lacking. The purpose of this

chapter is to describe the state-of-the-art with regard to sampling methodology for the detection, identification, and enumeration of Giardia cysts in water supplies. Many of the problems identified by Jakubowski and Ericksen (1979) still remain. Additional recent information will update that report where appropriate and the results of a study on multiple laboratory examination of seeded samples will be discussed and a summary of a workshop on Giardia detection methodology will be presented.

2. ESTIMATED LEVELS OF CYSTS IN SEWAGE AND WATER

In developing methods, some information on cyst levels that might be expected in sewage and water samples would be useful. Giardia is an intestinal parasite not likely to multiply in a water environment outside of a host. Tsuchiya (1931) studied cysts produced by two human carriers, A & B, for 36 and 28 days, respectively. An analysis of these data indicated a mean of 1.1×10^6 cysts/g of stool for subject A with a maximum observed level of 3.3×10^6 cysts/g. Mean daily production of cysts for subject A was 2.1×10^8 with a maximum of 7.1×10^8. Results for subject B were similar (mean of 1.6×10^6/g with a maximum of 4.3×10^6, and daily production of 2.0×10^8 with a maximum of 6.4×10^8). Tsuchiya did not report the subjects' age but it is assumed that they were adults.

Danciger and Lopez (1975) studied cyst production in 15 children three to seven years of age. Three patterns of excretion were noted: high with large numbers of cysts in nearly all stools; low, with small numbers of cysts in 40% of the stools; and mixed, where periods of high excretion alternated with shorter periods of low excretion and an average of about 60% positive stools. The number of cysts excreted ranged up to 2.2×10^6/g of formed stool with an overall mean of 5.8×10^5/g. Total daily production of cysts on a per capita basis was not reported.

Murray and Reeves (1977) estimated the per capita daily usage of water from public supplies in the United States in 1975 as 638 liters (168 gal). Water lost in the distribution systems and used for public activities such as firefighting, street cleaning, etc. was estimated at 36% or 230 liters (60 gal). Industrial and commercial use was 31% or 198 liters (52 gal). This results in an estimated residential usage of 210 liters (55 gal) per

person per day. Assuming that the average daily water usage of a family of four is 840 liters (220 gal) per day, and that 2.0×10^8 cysts are shed daily by an infected adult, the theoretical concentration of cysts in raw sewage would be 2.4×10^5 cysts/liter if one family member (25%) was shedding cysts. If 1% of the population were infected, the concentration of cysts in raw sewage would be 9.6×10^3 cysts/liter. Various surveys have indicated the prevalence of Giardia-infected individuals to be in the range of 1 to 24% (Healy, 1979). These calculations are also based upon a uniform dispersion of fecal material in sewage. Calculation of cyst levels in water supplies would then be dependent upon the degree of raw sewage contamination of a given water supply.

Sparkling mountain streams in pristine locales may be subject to contamination by wild animals in the absence of sewage discharges. The Camas, Washington outbreak (Kirner et al., 1978) and reports of infections in campers and hikers (Barbour et al., 1976) suggested that water supplies may become contaminated with Giardia by other than human fecal discharge. No signs of human fecal contamination were detected in the Camas outbreak and beavers were implicated as the most probable source of the Giardia organisms that produced the epidemic (Dykes et al., 1980). Frost et al. (1980) reported on a survey of Giardia prevalence in commercially trapped mammals. Up to 19% of the beavers and almost 43% of the muskrats were found to be positive for Giardia. It had been suggested by others that increased use of wilderness areas by recreationists might be a source of contamination of wildlife. However, Frost et al. found infected animals on watersheds where human use was restricted as well as on those open to recreational activity. This finding suggested that protected watersheds will not insure

a source water free of Giardia.

Using data developed in the animal survey, Frost (personal communication) calculated cyst levels in six Washington State streams on two watersheds. Factors included in the calculation were beaver population size, Giardia prevalence rate, stool size, number of cysts per gram of stool and stream flow rate. Using the minimum number of cysts detected per g of stool and the maximum stream flow rates, the density of cysts/gal in the streams ranged from 1.4×10^{-6} to 9.3×10^{-6}. However, when the maximum fecal cyst concentration was used in combination with the minimum flow rates of four of the streams (minimum flow data were not available for two of the streams), cyst densities ranged from 1.3 to 245/gal. These calculations indicated that a few infected beavers could heavily contaminate a small water system. These data further suggested the difficulty that would be encountered in trying to determine by water sampling alone, whether or not a given supply was at risk from Giardia contamination.

3. DETECTION METHODS

At the present time, there is no cultural method for detecting Giardia cysts in water samples. However, Meyer (1976) and others have cultivated the trophozoite stage, and the recent development of an excystation technique (Bingham and Meyer, 1979; Bingham et al., 1979; Rice and Schaefer, 1981) could conceivably lead to development of cultural methods. The available alternatives are animal feeding and direct microscopic examination of sample concentrates. Animal feeding experiments require special handling and isolation facilities and are time-consuming and expensive. Their application to surveys and monitoring would, therefore, be limited by economic and practical considerations. Animal feeding, however, is the only method for demonstrating the infectivity of Giardia cysts.

Methods employing microscopic detection in water sample concentrates are not quite as time-consuming as animal feeding but they are tedious. In addition, identification of the organism is dependent upon the training, experience, skill, and patience of the examiner. The results of a proficiency-testing program reported in 1977 by the Centers for Disease Control (CDC) indicated that between 20% and 35% of public and private laboratories could not correctly identify intestinal protozoa. Furthermore, the identification of protozoan cysts is not a skill that would be available in most water supply microbiological laboratories. In performing microscopic analysis it is also important to have a clean preparation as free as possible of inorganic and organic debris that could mask the presence of the organisms or interfere with identification. Nevertheless, recognizing these limitations, microscopic identification methods are the only currently available approach for detection of Giardia in water

samples.

3.1. Membrane Filter Methods

The basic assumption in the application of methods to the detection of Giardia cysts in water has been that some form of sample concentration is necessary. In 1956, Chang and Kabler described a membrane filter technique for concentrating Endamoeba histolytica cysts in tap water. Five replicate experiments were conducted at four cyst concentrations: 1, 3, 5, and 10 cysts/3.8 liters. Each sample was membrane filtered and the retained material was washed from the membrane with about 2 ml of distilled water. This concentrate was examined microscopically in a counting chamber. Mean cyst recoveries of 20%, 28%, and 42% were reported at cyst concentrations of 3, 5, and 10/3.8 liters, respectively. No cysts were recovered from the sample containing one cyst/3.8 liters.

During the investigation of the 1965-66 Aspen, Colorado giardiasis outbreak, Moore et al. (1969) adapted the Chang and Kabler method to examine water and sewage samples for Giardia cysts. Two-liter water samples from 10 sites throughout the city, and one-liter sewage samples from three main trunk lines were collected. The samples were each passed through cheesecloth, filtered through 0.45 µm porosity membrane filters, and the sediment on the filters was brushed into water, centrifuged, and preserved in 10% formalin for microscopic examination. No cysts were detected in the 10 water samples. Two sewage samples from lines draining the eastern section of the city contained three Giardia cysts and one or two cysts of Entamoeba coli and Endolimax nana per 100 ml.

Barbour et al. (1976), while investigating an outbreak in a group of campers, filtered 22 liters of stream water through a membrane filter.

Giardia cysts were not found but coccidian oocysts were observed in small numbers. With the exception of the Aspen sewage samples, the membrane filter method has not been successful in demonstrating cysts in water samples. Whether this is a function of the inability to process a sufficient volume of water, or of the failure to adequately "clean-up" the retained material, or perhaps indicates that the organism was not present in these water samples at the time of sampling, is not known.

3.2. Particulate Filter Methods

By the time of the Rome, New York outbreak in 1974-75, and apparently operating under the premise that larger volumes of water must be sampled to detect cysts, the CDC had developed a large-volume sampling technique (Shaw et al., 1977). The method used a swimming pool filter in which sand was the filtering medium. Ten samples of raw water, representing a total volume of about 1.1×10^6 liters (280,000 gal) were collected by pumping water through the filter at an average flow rate of 76 liters/min (20 gpm) daily for 10 days. Filter backwash was collected each day in two 210-liter (55 gal) drums and coagulated with alum. After a 24-hour settling period, the supernatant fluid was removed and 1-2 liters of sediment were collected. Aliquots of each sample were fed to beagle puppies, examined microscopically at Rome, and sent to CDC for further processing.

The sediment samples examined at CDC were washed through two layers of cheesecloth and an 80 µm wire screen by gravity, and then through a 50 µm porosity membrane filter by vacuum. Most of the supernatant was removed following centrifugation at 1,000 rpm for 10 min, and selected sediment samples were examined microscopically. Two of the ten samples fed to dogs produced infection 20 and 34 days after feeding, and a cyst was observed

microscopically at CDC in one of these samples. This was the first time that a cyst had been detected in a water supply, and the first time that concentrates from a water supply had been found infective for laboratory animals. The efficiency of this method has not been reported by CDC, but in one experiment where cysts were added to water at a level of 20/3.8 liters (one gal) and 34,200 liters (9,000 gal) were sampled, no cysts were detected by microscopic examination (B. Wood, personal communication).

Particulate filtration with diatomaceous earth as the medium has been investigated by the CDC (Juranek, 1979). Effective removal of cysts from seeded water samples was obtained but the cysts could not be separated from the diatomaceous earth particles thus precluding detection of cysts. A particulate filter method employing anionic and cationic exchange resins is currently under investigation in a U.S. Environmental Protection Agency (EPA) sponsored project. While good removals of cysts have been observed in preliminary experiments, the efficacy of this approach cannot yet be assessed.

3.3. Microporous Depth Filters

In 1976, EPA developed a concentration method involving filtration of a large volume (100 gal or more) of water through a microporous filter. The method has been described in detail elsewhere (Jakubowski and Ericksen, 1979; APHA, 1981). Briefly, concentration of cysts is accomplished by filtering the sample through the device shown in Figure 1. This device consists of an influent hose, filter holder, yarn-wound cartridge filter, effluent hose, water meter and a flow controller. The device is simply connected to a pressurized source and filtration is allowed to proceed until the requisite volume has been sampled. The filter is transported to the

laboratory on ice or under refrigeration and the filter fibers are extracted with distilled water to recover trapped particulate matter, including cysts. Depending upon the amount of particulate matter obtained and based more on art than on science, the filter extract is then subjected to a series of flocculations, sedimentations or screenings. The purpose of these steps is to further concentrate and purify the cysts from other particulate matter present in the extract.

In limited laboratory evaluation of the method (Jakubowski and Ericksen, 1979), the efficiency of cyst recovery was 6.3% (range, 3 to 15%). Analysis of the various steps involved in the method indicated 58% (range, 12 to 139%) recovery of cysts in the extraction process. More cysts were lost through 7 µm nylon screens than through 8 µm polycarbonate filters. Centrifugation was better than either screening or filtration when interfering particulates were not present. It was suggested that flocculation of the extract decreased recovery of cysts by more than one log.

In spite of processing problems and the apparent inefficiency of the method, it found widespread use in outbreak investigations and surveys almost as soon as it was developed. It was the first method successfully used to detect cysts in the distribution system of a community water supply and it was responsible for identifying a previously unsuspected new drinking water treatment plant as a source of contaminated water during the Berlin, New Hampshire outbreak (Lippy, 1978). The method has also been used to detect Giardia cysts in a variety of surface and well waters.

3.4. Primary Concentration versus Processing

All of the methodology discussed above may be broadly separated into two distinct stages: (1) primary concentration, and (2) processing,

including identification. The problems surrounding microscopic detection and identification of Giardia cysts are common to all of the methods. The cysts must be separated as much as possible from interfering debris in the sample and positive identification must be made by a well-trained individual. All of the methods have processing problems, i.e., difficulty in producing a final concentrate that would, ideally, contain all of the cysts present in the original sample and none of the interfering debris. All of the methods use a combination of flocculation, sedimentation, screening or filtration and centrifugation to accomplish this objective.

The principle difference in the methods concerns the manner of primary concentration. The assumption that a large volume of water must be processed in order to detect Giardia cysts is supported by the estimated levels of Giardia in sewage and in streams contaminated by infected beavers. If one accepts that assumption, then it is not surprizing that low-volume membrane filtration techniques have been unsuccessful. Further, if large volumes are required, then grab or batch sampling is not practical. For this reason, centrifugation has not been seriously considered as a primary concentration technique although at least one laboratory has used an algal centrifuge to process filter extracts and the adaptation of this centrifuge method to field sampling may be possible.

The CDC sampler has not been used in the field since 1976. Although it was the first sampler successful in detecting cysts in a water supply, it suffered from sheer size and related difficulties in transporting the equipment to the sampling site. In addition, collection of the sample was technically complicated. The EPA sampling device made it possible for anyone with the ability to make a garden hose connection and turn on a tap

to collect a Giardia water sample.

The ideal method would allow total recovery of all cysts in a given water sample through simple and inexpensive means; it would incorporate procedures that adequately purified the cysts from interfering debris; it would allow positive and rapid detection, identification and quantitation of the genus Giardia (and preferably, the species) in an objective manner, and it would provide information on the viability or infectivity potential of detected cysts. That method does not now exist.

4. MULTI-LABORATORY EVALUATION OF GIARDIA-SEEDED WATER SAMPLES

4.1. Background

In early 1980, the Colorado Department of Health initiated a Giardia survey in the Vail-Aspen-Steamboat Springs ski resort areas (Colorado Disease Bulletin, 1981). In an earlier outbreak investigation, Giardia cysts had been detected in finished water samples from communities not experiencing an outbreak at the time as well as from different points in the water supply involved in the outbreak. The objective of the 1980 study was to determine if a dose-response relationship could be established by quantitating cysts detected in drinking water samples concentrated with the EPA filter device. These data would then be correlated with occurrence of cases in tourists in the study areas. Although the EPA methodology has not been proposed for routine monitoring or for acquisition of quantitative data, the investigator felt that the method would be applicable considering the numbers of cysts that had been detected in various water supplies during the earlier outbreak investigation.

Shortly after the study was initiated, the investigator sent portions of a filter extract to three laboratories for Giardia examination. One laboratory reported finding Giardia cysts and the other two laboratories reported the sample as negative. Considering our own experience and that of other laboratories, and since the methodology has not been standardized, these results might have been expected. However, the investigator was understandably concerned about interpretation of the data being gathered in the dose-response study. It was decided to send water sample filter extracts seeded with different levels of Giardia cysts to each of five laboratories that had experience in processing such samples. In order to

assure that each laboratory use its normal routine processing procedure for samples of this type, it was necessary that the individuals doing the processing not be informed that these were seeded or special samples.

4.2. Protocol

4.2.1. Cysts

The EPA laborary in Cincinnati prepared three coded vials as follows:

Vial #1 - 250,000 cysts/50 ml

Vial #2 - 0 cysts/50 ml

Vial #3 - 25,000 cysts/50 ml

The cysts had been obtained one week earlier from a stool sample provided by an asymptomatic carrier. The cysts had been purified by sucrose flotation and distilled water washing. The cysts in the three vials were suspended in distilled water containing 100 µg/ml gentamicin to prevent bacterial growth. The coded vials were shipped to Colorado on cold-paks and they were received at the Department of Health laboratory within 24 hours of shipping.

4.2.2. Water Sample Filter Extracts

Two types of water sample filter extracts were prepared: "clean" and "dirty", subjective designations relating to the amount of particulate matter collected on the filters. All water sample filter extracts were prepared by a single laboratory at the Colorado Department of Health. The "clean" sample represented the pooled particulates collected from four EPA filter device samples obtained from two water supplies. The "dirty" sample contained particulates present on five filters collected from three different water supplies. Filter extracts were prepared by cutting the orlon filter fibers from the support core of the 10 inch yarn-wound filter cartridges and washing the fibers by hand in distilled water.

4.2.3. Preparation and Shipment of Giardia-Seeded Filter Extracts

The "clean" and "dirty" filter extracts were each divided into three equal portions and a predetermined volume of the coded cyst preparations was added to each portion. Consequently, there were six samples as follows:

Giardia Cysts/250 ml	
"Clean"	"Dirty"
0	0
1,000	1,000
10,000	10,000

Five sets of six samples, with 250 ml in each sample, were prepared, packed in commercial canned ice and shipped to five laboratories. All laboratories received their samples within about 48 hours of shipping. Two laboratories reported that the samples were cold upon arrival; the other three laboratories reported that the samples were at room temperature upon receipt.

4.3. Results and Discussion

The results of the analysis of the six samples by the five laboratories are shown in Table I. In the "clean" samples, all laboratories had negative findings for the extracts to which cysts had not been added. For the clean samples containing 1000 cysts/250 ml, 4/6 (67%) of the determinations were positive. Cysts were detected by all laboratories that used a $ZnSO_4$ flotation purification technique. Cysts were not detected by the laboratory using a sucrose flotation technique nor by the observer in laboratory 5 (5B) who used a membrane procedure. The number of cysts detected in these samples ranged from 27 to 100 (2.7 to 10% of the number added to this set of samples). For the "clean" samples containing 10,000

cysts/250 ml, all laboratories reported positive results and the numbers of cysts ranged from 70 to 37,500 (0.7% to 375% of added cysts).

Only one laboratory (No. 1) reported finding cysts in a "dirty" sample to which cysts had not been added. The presence of indigenous cysts in that sample could not be discounted. Cysts were found by three laboratories (50% of the determinations) in "dirty" samples containing 1000 cysts/250 ml with numbers detected ranging from 3 to 100 (0.3 to 10%). As with the "clean" samples, all laboratories detected cysts in the "dirty" samples containing 10,000 cysts/250 ml. The numbers detected ranged from 10 to 12,500 (0.1% to 125% of added cysts).

The results of this study clearly indicate that the processing methods used cannot be considered quantitative by any appropriate criteria. It would be impossible to relate the numbers of cysts detected in one laboratory to the numbers detected by another laboratory in the same or a different sample and establish the true concentration of cysts in the original sample with any degree of accuracy.

It should be emphasized that this study concerned only the processing method, i.e., the ability to detect cysts in an extract prepared from samples collected with the EPA filter device. It did not address the variability that might be expected in preparing the extract from a filter cartridge. Even so, this study did provide some insights into the sensitivity and usefulness of the procedures as a qualitative tool. All laboratories detected cysts in both the "clean" and "dirty" samples when the extracts contained 10,000 cysts/250 ml. When the samples contained 1000 cysts/250 ml, cysts were detected in 50% and 67% of the "dirty" and "clean" determinations, respectively.

Jakubowski and Ericksen (1979) found the recovery of cysts from the orlon filter to range from 12 to 139% with a mean of 58%. If it is assumed that 50% of the cysts collected by the filter will be recovered in the extract, then it might be reasonable to expect the results to be positive in 50-67% of the determinations when a volume of water containing 2000 cysts is filtered.

Four of the laboratories involved in this study used a $ZnSO_4$ flotation technique for concentrating and purifying the cysts. The procedures varied from one laboratory to another in terms of specific gravity of the $ZnSO_4$ solution and centrifugation force and time. There was no clear-cut indication that the variations employed by one laboratory were superior to those employed by other laboratories. Laboratory No. 2 employed a sucrose flotation technique that was the most accurate in detecting and enumerating cysts when they were present at a concentration of 10,000/250 ml. However, the sucrose technique was the only one not successful when the sample contained 1000 cysts/250 ml. The reason for this insensitivity is not known.

Two of the laboratories employed direct microscopic examination of the samples before doing further processing. Laboratory No. 4 correctly identified all four positive samples with this technique. Laboratory No. 1 correctly identified the two samples containing the higher concentration of cysts. These results would indicate that examination of filter extracts prior to further processing would be a useful procedure, especially if time is of importance, e.g., in an outbreak investigation.

5. RECOMMENDATIONS OF A *GIARDIA* METHODS WORKSHOP

5.1. Background

With the information from the five-laboratory evaluation available and recognizing the problems associated with interpretation of occurrence data that was being obtained in various studies, the EPA convened a workshop on *Giardia* methodology in Cincinnati in September, 1980. The goals of the workshop were to:

A. Identify the best available methodology;

B. Develop recommendations for sample collection, transport, processing and examination;

C. Establish minimum criteria for *Giardia* cyst identification; and

D. Develop recommendations on interpretation of positive and negative findings in water supplies.

Representatives from all five laboratories that cooperated in the seeded study participated in the workshop. The participants were: W. Brewer, Wright State University; F. Frost and R. Holman, Washington State Department of Social and Health Services; G. Healy, Centers for Disease Control; C. Hibler, Colorado State University; E. Meyer, Oregon Health Sciences University; T. Ericksen, W. Jakubowski, E. Rice, J. Sauch and F. Schaefer, USEPA.

5.2. Issues and Recommendations

During the two-day workshop, the participants reviewed the results of the five-laboratory study, discussed procedures used in each of their laboratories for examination of *Giardia* water samples, identified issues and developed recommendations on these issues, and agreed on a reference method. The method is presented in detail later in this chapter.

Consensus recommendations were developed on each of the issues, as follows:

A. Reference Method. It was agreed that a reference method should be suggested. It was recognized that any proposed method would be based in large part on opinions rather than on hard data and that there would be shortcomings in such a method. However, it was agreed that suggesting a method would promote uniformity among laboratories and provide a basis for comparing results. Alternate methodology should be compared to the reference method for precision, accuracy, and sensitivity.

Recommendation: The precision, accuracy and sensitivity of the various procedures in the reference method should be determined. Reliable cyst counting procedures to determine input and recovery should be developed. The suitability of Giardia muris cysts as a model for Giardia lamblia in method evaluation should be determined. Surfactants and other compounds should be evaluated for elution of cysts from filters. The conditions for zinc sulfate and sucrose purification of cysts from concentrates should be determined. The formalin-ether method for recovering cysts from water sample concentrates should be evaluated and compared.

B. Giardia Cyst Identification. Identification of cysts requires microscopic examination of concentrates by competent, well-trained individuals with a demonstrated proficiency for the diagnosis of intestinal protozoa.

Recommendation: Suspect organisms should possess the proper size and shape and at least two internal characteristics (nuclei, axostylar rods, parabasal bodies).

C. Giardia Cyst Viability. Viability as determined by staining procedures has not correlated with that determined by excystation. The ability of excystation to indicate viability of cysts from water samples is questionable because obtaining a sufficient number of cysts to perform the test is unlikely. Although SPF beagle puppies have been used for determining infectivity of Giardia cysts, obtaining and maintaining these animals for this purpose is impractical.

Recommendation: In waterborne outbreaks where concurrent epidemiologic data implicate drinking water as the vehicle of transmission, it is not necessary to determine the viability of cysts detected in drinking water samples.

D. Immunological Methods. Microscopic examination and identification of cysts in water samples is a tedious and demanding task. It is often difficult or impossible to positively identify cyst-like structures as Giardia.

Recommendation: Immunological methods (fluorescent antibody staining, ELISA) hold considerable promise for improving cyst detection and identification capabilities and their investigation is encouraged.

E. Application of Methods. The existing methodology has not been standardized nor sufficiently evaluated.

Recommendation: Existing methodology appears suitable for investigation of outbreaks and for special research applications where concurrent epidemiological data are collected. The methodology is not currently suitable for routine monitoring or surveillance application.

F. Animal Models and Sources of Giardia Cysts. There is no existing economical, reproducible, and easy to maintain animal host for Giardia lamblia. Cysts cannot currently be produced in culture. Experimental work with G. lamblia must rely on identifying human sources and purifying cysts from feces.

Recommendation: Identification of a suitable laboratory animal host for G. lamblia (perhaps the rat or inbred strains of mice) is encouraged. Attempts should proceed to develop cysts from trophozoites in culture. Trapping activities and kennels may provide adequate interim sources of animal cysts. Human cyst-containing material may be obtainable from private and public laboratories, medical practitioners, stool surveys, and day-care centers.

G. Animal Surveys and Cross-Species Infectivity. Determining water supplies at risk solely by examination of water samples for cysts is not practical because of a lack of data on method performance and due to an inability to interpret either a positive or a negative finding. Beavers have been implicated in waterborne outbreaks of giardiasis and cysts from beavers have been found infective for man and other

animals. Beavers and muskrats, because of their habits and relative abundance on watersheds, are the most likely source of non-human Giardia contamination of a water supply.

Recommendation: The best available procedure for determining if water supplies are at risk appears to be through watershed animal stool surveys, especially beavers and possibly muskrats. Additional characterization of muskrat cysts is needed to determine their potential for causing human infection.

5.3. Reference Method. As stated previously, many of the steps in the Reference Method have not been verified under controlled laboratory conditions but represent the consensus of the group and they are based on experience and professional judgement. A flow chart for the method is shown in Figure 2.

A. Sampling Points. Samples should be collected at the raw water source, at the treatment plant prior to entering the distribution system, and prior to disinfection if possible, and in the distribution system. The total number of samples collected will depend upon the objective of the investigation and on the resources available.

B. Sample Collection. A minimum sample size of 380 liters (100 gal) is suggested. A continuous flow microporous filter device is recommended. The device (Figure 1) should consist of an influent hose, filter holder, filter element, effluent hose, and water meter. If a pressurized source is

not available, a suitable pump and source of power will be required. A flow control device that will allow sampling at a rate of 3.8 lpm (1gpm) at a pressure of 15-18 psi is recommended. A filter holder that will accept a 10 inch yarn-wound cartridge is recommended. The filter cartridge should have a nominal porosity rating of 1 µm and should consist of orlon fibers. Other suitable filter materials may be used if they do not release fibers into the concentrate during processing. The filter holder should be connected such that the flow of water is from the outside to the inside of the filter cartridge. After the required sample volume has been collected, disconnect the filter unit and drain residual water from the effluent end of the filter holder. Aseptically remove the filter cartridge and place it and any remaining residual water in a plastic bag or leakproof container. If additional samples are to be collected with the same filter device, thoroughly rinse the influent hose and filter holder with the water to be sampled before installing another filter cartridge.

C. Sampling Data. The following information should be collected and attached to each sample: Water source (raw or treated); sampling point, time, and date; volume filtered; flow rate and pressure; name and phone number of individual collecting the sample; and the sample number. Ancillary data that should be collected on the sample include pH and turbidity of the water, bacteriological results, and free

and total disinfectant levels.

D. Sample Transport and Storage. Filter samples should be placed under refrigeration or wet ice immediately after collection. Do not freeze the samples. When received in the laboratory, samples should be stored under refrigeration until processed. Shipping and storage times should be minimized as much as possible.

E. Sample Processing.

1. Extraction. Filter samples should be handled aseptically. Using a razor knife, separate the filter fibers from the support core by making a longitudinal cut down the entire length of the filter cartridge. Cut the fibers into two or more approximately equal portions consisting of an outer and an inner layer. There will often be a fairly distinct line of demarcation based on the depth to which particulate matter from the water sample has penetrated the filter cartridge. An alternative method of removing the fiber from the core is to locate the end of yarn on the outside of the cartridge, unwind the fiber, and divide into equal portions. Wash each portion separately with 1 liter of distilled water by kneading or by shaking for approximately 10 min. Express the fluid from the fiber portions and combine all of the fluid. If the

fiber mat retains significant amounts of particulate matter, the extraction process on those portions may be repeated. If further processing is not possible at this point, the extract may be preserved by adding a sufficient volume of 37% V/V formaldehyde to produce a final concentration of 2% V/V formaldehyde. Refrigerate the preserved extract until concentration procedures can be performed.

2. Extract Concentration. The filter extract may be concentrated by allowing the extract to sediment in the refrigerator overnight or by centrifuging the extract at 600 xg for 5 min. If available, an algal centrifuge may be used for this step. Decant or aspirate the supernatant fluids and resuspend the pellets in a volume of 2% V/V formaldehyde equal to the total volume of the combined sediments. If little or no sediment is present, suspend the sediments in 10 ml of 2% V/V formaldehyde. (The algal centrifuge produces a total volume of 7 ml of supernatant fluid and sediment. Add an additional 3 ml of 2% V/V formaldehyde and wash by centrifugation.) Recentrifuge the combined sediments for 5 min at 600 xg. If the sediment volume is $\leq$1 ml, continue processing by decanting or aspirating the entire

supernatant. discard the supernatant fluid. If the sediment volume is >1 ml, resuspend the sediment in the supernatant fluid, transfer a volume of suspension equivalent to 1 ml of sediment to a 15 ml tube and again centrifuge for 5 min at 600 xg. Decant and discard the supernatant fluid.

Add 2-3 drops of Lugol's Iodine (dilute stock solution 1:5 with sp.g. 1.20 zinc sulfate solution) to the pelleted material and mix with an applicator stick. Add 5 ml of zinc sulfate solution (sp.g. 1.20) and mix on a vortex mixer. Bring the volume up to the top of the tube with additional zinc sulfate solution being careful not to allow spillover. Using a swinging bucket rotor, centrifuge for 3 min at 650 xg. Touch a clean, grease-free, #1 22 x 22 mm coverslip to the meniscus after allowing the tube and contents to remain static for 2 to 3 min after centrifuging. Be careful to handle the coverslip by the edges. Carefully lift the coverslip straight up, place on a slide, and seal the coverslip with vaspar.

3. Microscopic Examination. Scan the entire coverslip area with a 10X objective on a bright-field microscope. The entire area between the cover-

slip and the slide should be examined. Suspect organisms should be identified using a 45X objective and an ocular micrometer. Identification must be made by an individual with a demonstrated proficiency for recognizing and differentiating intestinal protozoa. Care should be taken not to confuse Giardia with yeasts, diatoms, coccidia, and other organisms. Positive identification of Giardia requires observing structures of the right size and shape and having at least two internal anatomic characteristics. Sediment from the entire sample must be examined in the above manner before a sample can be declared negative.

F. Reporting of Results. It is recommended that measurements be made and reported of Giardia cyst-like structures larger than 8 µm and less than 19 µm. In addition, any internal morphological features observed should be reported. Other non-Giardia microorganisms detected should also be recorded and reported.

5.4. Discussion

The goals of the workshop were attained and agreement was unanimous on most issues. On the issue of Giardia identification, there was a minority opinion that the detection of cyst-like structures of the proper size and shape and the observance of one, instead of two, internal structure would suffice to establish identification. However, the consensus was that the

potential for confusion of Giardia cysts with other organisms was such that the discernment of two internal characteristics should be required to prevent misidentification. If these requirements cannot be satisfied, then the report should state that "Giardia-like" cysts were observed and the characteristics of the cysts should be indicated.

There was also a difference of opinion on processing filter extracts containing large volumes of sediment. The minority opinion held that the examination of samples was tedious and time-consuming and that it should not be necessary to examine all of the sediment from a sample before declaring it positive or negative. The majority agreed that, to declare a sample positive, only one cyst had to be identified and, at that point, it would not be necessary to examine any remaining sediment. However, a negative declaration could not be made unless all of the sediment from a given sample was examined. If less than all of the sample is examined, then the volume of the original sample should be adjusted accordingly in the report. For example, if 380 liters (100 gal) of water is filtered and the extract produces 10 ml of sediment but only 1 ml is examined microscopically and found negative, then only a 38 liter (10 gal) portion of the original water has been analyzed. It cannot be stated that the 380 liter (100 gal) sample was negative.

6. STATE-OF-THE-ART

Giardia detection methods for water samples have at least four potential applications: 1) monitoring of water supplies, 2) evaluation of water treatment processes, 3) determination of supplies at risk, and 4) outbreak investigations.

The term "monitoring" is used here in the context of analysis of a sample to see if it satisfies some standard. A standard for *Giardia* in water does not now exist and it is difficult to envision the establishment of such a standard. It seems more likely that treatment requirements would be suggested rather than limits set on the numbers of *Giardia* allowable in water. Even if a quantitative standard were in effect, compliance could not be determined with existing methodology. As shown earlier, the available methodology is not quantitative, but more importantly, the microscopic detection of a *Giardia* cyst does not provide information on the viability or infectivity of the detected cyst.

Evaluating the efficacy of water treatment processes for the removal of *Giardia* cysts also requires the use of quantitative methods. At least two laboratory and pilot scale studies have been undertaken to determine the efficiency of drinking water treatment for *Giardia* removal (Logsdon, *et al.*, 1981; Luchtel, *et al.*, 1980). These studies used *G. lamblia* or *G. muris* cysts or radioactive beads as cyst models and the yarn-wound filter method was deemed inappropriate because of low efficiency and variability. Filtration through 5 μm polycarbonate filters was used to obtain quantitative data and the efficiency of the membrane filters for recovery of cysts ranged from 45 to 75%. The membrane filters were suitable for this application because large numbers of cysts were dosed into the systems and

relatively clean water was being sampled. However, the disadvantages of membrane filters described earlier would make the use of these filters in field studies on occurrence of cysts or in outbreak investigations less desirable than the use of the yard-wound filter cartridge.

With the attention given to waterborne outbreaks of giardiasis in recent years, a number of public and private laboratories have been besieged with requests to analyze water samples for Giardia. In isolated cases of giardiasis or when cases are limited to a family unit, physicians appear to be more frequently implicating the patients' water supply as the vehicle for transmission. This results in requests to the utility or to the health department to ". . . check the water for Giardia." While drinking water can and has undoubtedly served as a source of infection in outbreak situations, its role in maintaining an endemic level of infection has not been clearly established. Person-to-person transmission occurs and has been documented in day-care centers (Black, et al., 1977, 1981) and a foodborne outbreak of giardiasis has been reported (Osterholm, et al., 1981). In the undisinfected water supply, the coliform test may be a suitable indicator for the potential presence of Giardia. The cysts are not likely to be present in these supplies in the absence of coliforms if the contamination is from either human or animal sources. Additional information may be gathered by conducting a sanitary survey of the supply. Problems detected should be corrected whether or not the water can be established as the vehicle of transmission.

The problem is more complex in chlorinated or disinfected water supplies since Giardia cysts are more resistant to inactivation than are coliforms (Jarroll, et al., 1981) and the coliform test may therefore not

an appropriate indicator. In addition to sanitary surveys, the potential risk of contamination of these supplies may be evaluated as suggested by the workshop participants through a stool survey of wild animals, especially beavers and muskrats, inhabiting the watershed.

The sensitivity and reproducibility of available methodology for detecting cysts in water has not been adequately established or determined with a variety of waters. Using the method to evaluate the potential risk associated with a given water supply is therefore of doubtful value. Also, in undisinfected as well as disinfected supplies, the infectivity of detected cysts may be difficult or impossible to determine. The laboratory contemplating analysis of water for Giardia should also consider cost, time and personnel requirements. Only standard laboratory equipment and facilities are needed. The filter device is relatively inexpensive and can be constructed at a cost of approximately $125. Reagents and materials per sample would amount to approximately five dollars, most of which would be the cost of the filter cartridge. The extraction of the filter, processing of the extract and preparation of the microscope slides should pose no difficult problems for most biological technicians. However, the examination of the slides for detection and identification requires specific competence certainly not available in most water laboratories. Also, depending upon the amount of sediment in a single sample it could easily take two or more days to examine a single sample before declaring it negative. The purpose of the sampling, the interpretation of the results and the available resources should therefore be carefully considered before embarking on a program of Giardia water sampling.

Although poorly characterized and not perfect, the suggested Reference

Method has its greatest potential usefulness in the investigation of waterborne outbreaks. There have been some successes and some failures. The failures may have been due to insensitivity of the concentration and detection procedures or to intermittent or one-time contamination events of the water supply. The 15th edition of Standard Methods for the Examination of Water and Wastewater (APHA, 1981) contains a tentative method for Giardia. Although published in 1981, the method was first written in 1978 and it is suggested that the Reference Method, which will be submitted with any interim modifications for publication in the 16th edition of Standard Methods, be used instead.

Proposing the Reference Method is a beginning and not an end. The intent was to provide improvements through experience of a method which has been successful in certain applications and to allow a basis for developing further improvements and modifications. New and innovative approaches are needed in the primary concentration of Giardia cysts from water, in purifying the cysts from interfering debris, in procedures for detecting and identifying the cysts and in determining their infectivity. It is hoped that the availability of a reference method will stimulate research in these areas.

7. ACKNOWLEDGMENT

The assistance and cooperation of Dr. Sandra Lapham, formerly of the Colorado State Health Department in setting up and conducting the multi-laboratory seeded study, and of the workshop participants in developing issues, recommendations and the Reference Method, is sincerely appreciated and gratefully acknowledged.

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Table I. Recovery of Seeded G. lamblia Cysts From EPA Water Filter Extracts

Sample	No. of Cysts Seeded/250 ml	No. of Cysts Reported/250 ml by Five Laboratories[a]					
		1	2	3	4	5A	5B
'Clean'							
1	0	0(-)[b]	0	0	0(-)[b]	0	0
2	1000	100(-)	0	50	27(+)	75	0
3	10,000	1600(+)	37,500	810	70(+)	120	750
'Dirty'							
1	0	50(-)	0	0	0(-)	0	0
2	1000	100(-)	0	0	3(+)	0	83
3	10,000	400(+)	12,500	10	46(+)	15	250

[a]Laboratory 2 used sucrose flotation for purification and further concentration; Laboratories 1, 3 and 4 used $ZnSO_4$ flotation; Laboratory 5 split the samples using $ZnSO_4$ flotation for one (5A) and membrane filtration for the other (5B).

[b]Signs in () indicate if sample was positive or negative upon direct examination.

Figure 1. USEPA *Giardia* Sampling Device.

A - inlet hose; B - filter housing;

C - outlet hose; D - water meter;

E - limiting orifice flow controller.

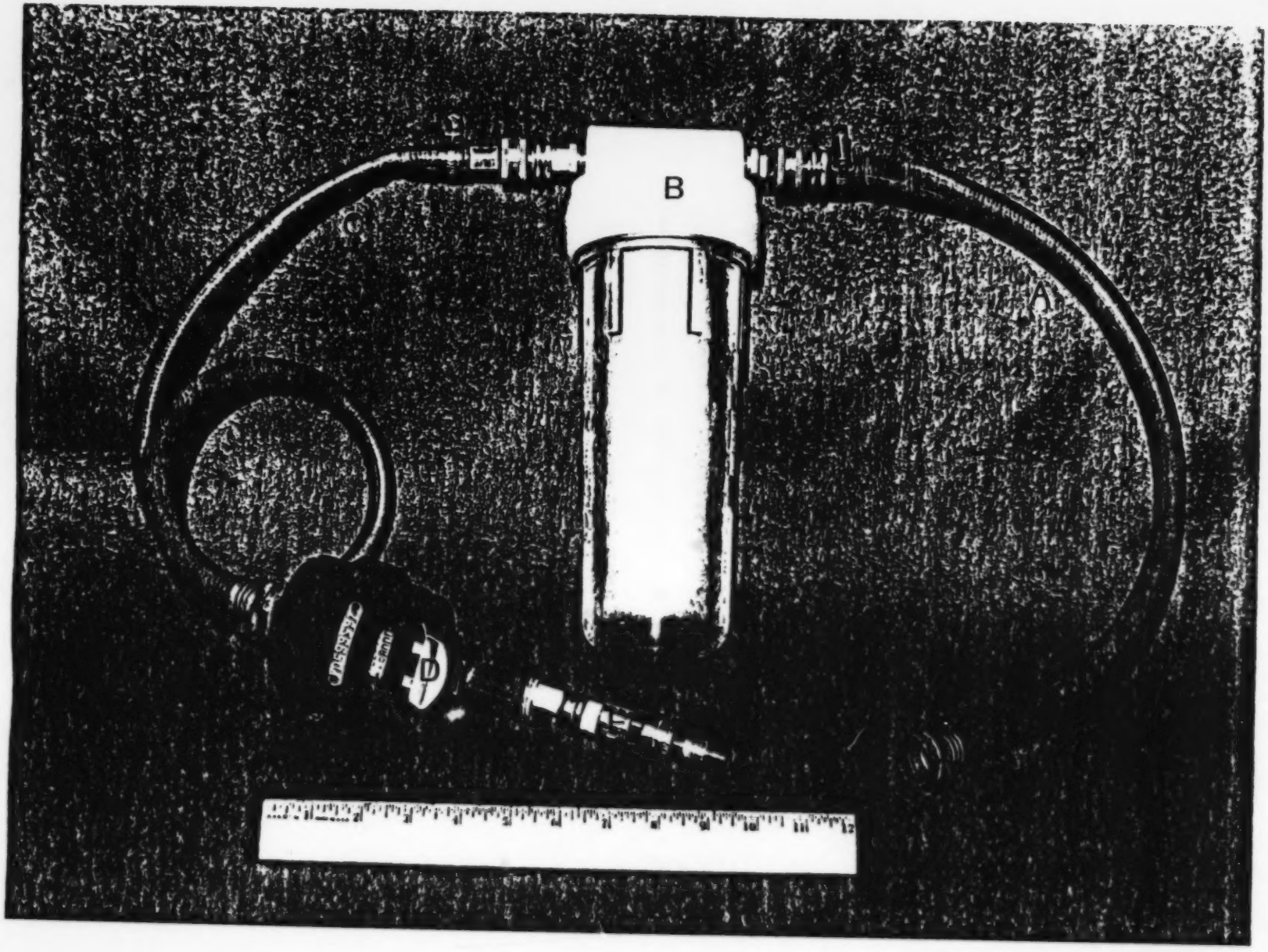
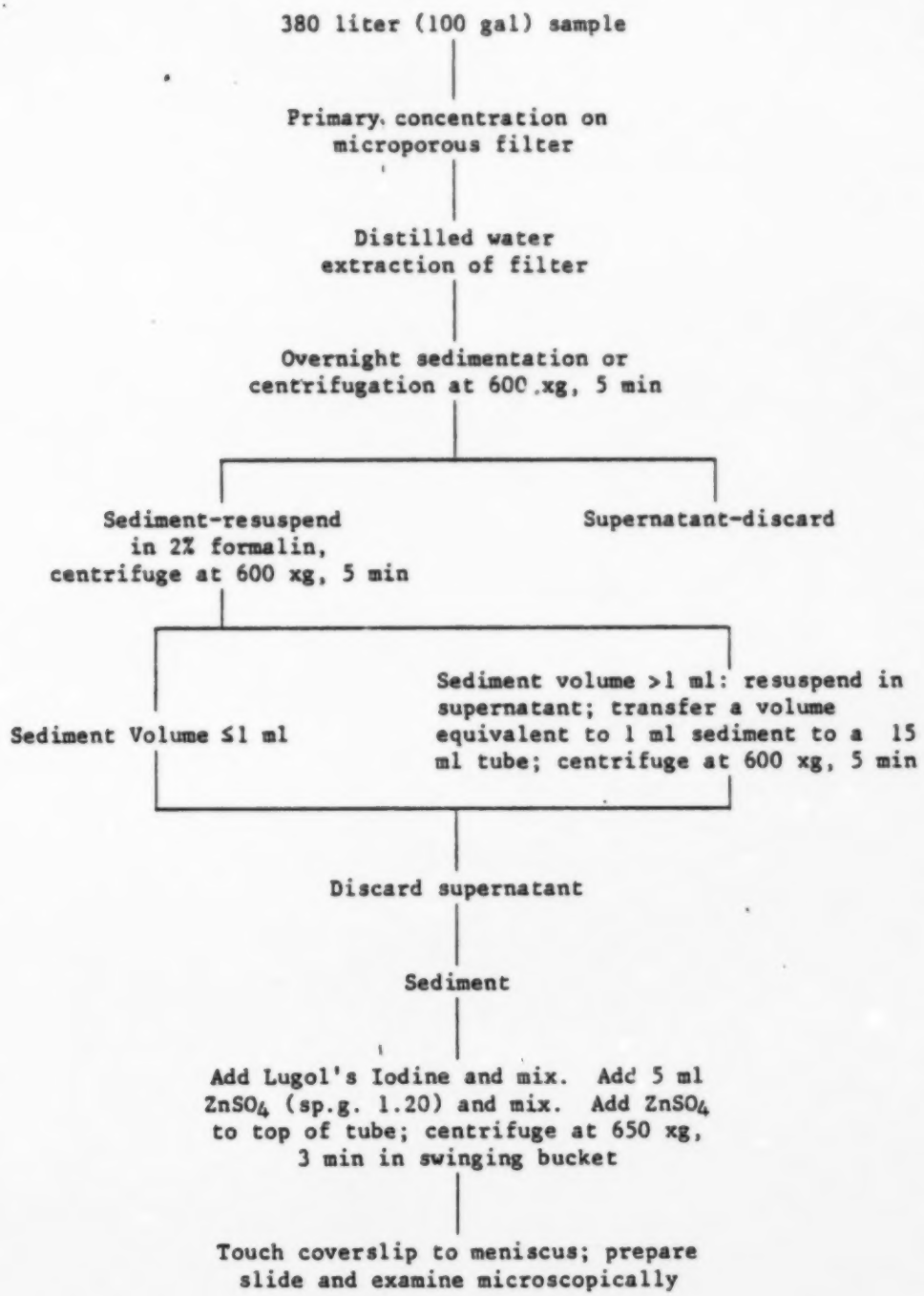

B
A

Figure 2. *Giardia* Reference Method Flow Chart


380 liter (100 gal) sample
Primary concentration on microporous filter
Distilled water extraction of filter
Overnight sedimentation or centrifugation at 600 xg, 5 min
Sediment-resuspend in 2% formalin, centrifuge at 600 xg, 5 min
Supernatant-discard
Sediment Volume ≤1 ml
Sediment volume >1 ml: resuspend in supernatant; transfer a volume equivalent to 1 ml sediment to a 15 ml tube; centrifuge at 600 xg, 5 min
Discard supernatant
Sediment
Add Lugol's Iodine and mix. Add 5 ml $ZnSO_4$ (sp.g. 1.20) and mix. Add $ZnSO_4$ to top of tube; centrifuge at 650 xg, 3 min in swinging bucket
Touch coverslip to meniscus; prepare slide and examine microscopically